Biochem. J. (1975) **145**, 185–199
Printed in Great Britain

Separation by Thin-Layer Chromatography and Structure Elucidation of Bilirubin Conjugates Isolated from Dog Bile

By KAREL P. M. HEIRWEGH, JOHAN FEVERY, ROGER MICHIELS, GUSTAAF P. VAN HEES and FRANS COMPERNOLLE
Department of Liver Physiopathology, Rega Instituut, Universiteit te Leuven, Leuven, Belgium, and Department of Macromolecular and Organic Chemistry, Universiteit te Leuven, Heverlee, Belgium

(*Received* 26 *July* 1974)

1. A system for separation of bile pigments by t.l.c. and for their structure elucidation is presented. Separated bile pigments are characterized by t.l.c. of derived dipyrrolic azopigments. 2. At the tetrapyrrolic stage hydrolysis in strongly alkaline medium followed by t.l.c. demonstrates the presence of bilirubin-IIIα, -IXα and -XIIIα and allows assessment of their relative amounts. 3. Most structural information is derived from analysis of dipyrrolic azopigments. Such derivatives, obtained by treatment of separated bile pigments with diazotized ethyl anthranilate, were separated and purified by t.l.c. Micro methods showed (*a*) the nature of the dipyrrolic aglycone, (*b*) the nature of the bonds connecting aglycone to a conjugating group, (*c*) the ratio of vinyl/isovinyl isomers present in the aglycone and, (*d*) the nature of the conjugating groups (by suitable derivative formation and t.l.c. with reference to known compounds). 4. In bile of normal dogs at least 20 tetrapyrrolic, diazo-positive bile pigments could be recognized. Except for two pigments the tetrapyrrolic nucleus corresponded predominantly to bilirubin-IXα. All conjugated pigments had their conjugating groups connected in ester linkage to the tetrapyrrolic aglycone. Apart from bilirubin-IXα, monoconjugates and homogeneous and mixed diconjugates of bilirubin were demonstrated; conjugating groups of major importance were xylose, glucose and glucuronic acid. 5. Bilirubin isomer determination on native bile and isolated bile pigments, and dipyrrole-exchange assays with [$^{14}C_8$]bilirubin indicated (*a*) that the conjugates pre-exist in bile, and (*b*) that no significant dipyrrole exchange occurs during isolation of the pigments.

In general, for structure elucidation of bilirubin pigments, formation and analysis of the more stable, dipyrrolic azo-derivatives has been preferred (Billing *et al.*, 1957; Schmid, 1957; Talafant, 1956; Heirwegh *et al.*, 1973*a*). Structure determination of these azopigments identifies a dipyrrole half with or without a conjugating group derived from the parent tetrapyrrole (e.g., see Fig. 2 of Fevery *et al.*, 1972*a*). The procedure is valid provided neither fragments are lost nor changes occur during formation or purification of the azopigments. Without preceding isolation of the tetrapyrrole, elucidation of its structure is possible only when (*a*) a single azopigment, or (*b*) mixtures of unconjugated and of one type of conjugated azodipyrrole are obtained. This condition applies when di- and/or monoconjugated bilirubin is synthesized *in vitro* (Heirwegh *et al.*, 1973*b*); it appears to be closely verified for normal rat bile (Heirwegh *et al.*, 1970). Therefore, in general, separation at the stage of the tetrapyrrolic pigments preceding azopigment analysis is required.

The classical work of Cole *et al.* (1954) and of Billing *et al.* (1957) on the column chromatographic separation of diazo-positive bile pigments demonstrated three bands presumed to represent bilirubin, and its mono- and di-glucuronide. Recent disclosure of a variety of conjugating groups (Kuenzle, 1970*b*; Compernolle *et al.*, 1971; Fevery *et al.*, 1971) has indicated that many more kinds of conjugated bilirubins must exist. The existence of bilirubin monoglucuronide, postulated by Billing *et al.* (1957) and firmly supported by recent work (Ostrow & Murphy, 1970; Jansen & Billing, 1971), was confirmed and the existence of other monoconjugates established (see below).

In the present work t.l.c. of diazo-positive bile pigments was investigated. Diazo-coupling techniques were used to identify and determine the structures of the separated tetrapyrroles. Attempts were made to adapt the assays to the micro scale. Normal dog bile was taken as a model source of bile pigments, as analysis of the azopigments has previously demonstrated predominance of a relatively small number of stable, well-characterized conjugating groups (Fevery *et al.*, 1971; Table 1).

The Greek letter notation, adopted by Heirwegh *et al.* (1970) to indicate azo-derivatives isolated by t.l.c., has been used in the present work. For example, with this symbolism azodipyrrole and its xylopyranoside are denoted as azopigment α_0 and α_2

Table 1. *Trivial names and partial structures of chromatographic reference azopigments*

Dipyrrolic azopigments obtained from the sources indicated in parentheses have been structurally characterized (see the references). In the text they are denoted by Greek letters (Heirwegh *et al.*, 1970; Fevery *et al.*, 1971, 1972*b*). When unknown azopigments are indicated by the same letters this implies the same chromatographic mobility as the corresponding reference compounds, but not structural identity.

Trivial name	Structure	References
Azopigment α_0	Azodipyrrole (bilirubin)	Compernolle *et al.* (1970, 1971); Jansen & Stoll (1971)
Azopigment α_{0M}	Azodipyrrole carboxylic acid methyl ester	Compernolle *et al.* (1970); Jansen & Stoll (1971)
Azopigment α_{0N}	Azodipyrrole carboxylic acid amide	Compernolle *et al.* (1970)
Azopigment α_2	Azodipyrrole (β?)-D-xylopyranose (dog bile)	Compernolle *et al.* (1971); Fevery *et al.* (1971)
Azopigment α_3	Azodipyrrole β-D-glucopyranose (dog bile)	Compernolle *et al.* (1971); Fevery *et al.* (1971)
Azopigment δ	Azodipyrrole β-D-glucopyranuronic acid (rat bile)	Heirwegh *et al.* (1970); Compernolle *et al.* (1970)
Azopigment δ	Azodipyrrole β-D-glucopyranuronic acid (from enzyme incubation mixtures)	Fevery *et al.* (1972*a*)

Table 2. *Diazo-positive bile pigments isolated from dog bile by t.l.c.*

The components, except for those in parentheses, were detected in all bile samples analysed. The degree of certainty of identification applicable to the components is discussed in the text. Some of the structures are shown in Scheme 1.

Band no.	Formula	Structural information
1	$\bar{\alpha}_0$–$\bar{\alpha}_0$	Bilirubin-IXα
2	–	Related to mesodihydrobilirubin
3	($\bar{\alpha}_0$–$\bar{\alpha}_{0M}$)	Presumably monomethyl ester of bilirubin-IXα
4	($\bar{\alpha}_0$–$\bar{\alpha}_1$)	Presumably an ester monoconjugate of bilirubin
5	$\bar{\alpha}_0$–$\bar{\alpha}_x$	Ester monoconjugate of bilirubin-IXα; the conjugating group contains hydroxyl and/or amino group(s)
6	($\bar{\alpha}_x$–$\bar{\alpha}_x$)	Only azopigment α_x was obtained; presumably an ester dicongujate of bilirubin
7	$\bar{\alpha}_0$–$\bar{\alpha}_2$	Ester monoxyloside of bilirubin-IXα
8	$\bar{\alpha}_1$–$\bar{\alpha}_1$	Ester diconjugate of bilirubin-IXα; the conjugating group contains hydroxyl and/or amino group(s)
9	$\bar{\alpha}_0$–$\bar{\alpha}_3$	Ester monoglucoside of bilirubin-IXα
10	($\bar{\alpha}_3'$–$\bar{\alpha}_3'$)	Only azopigment α_3' was obtained: presumably an ester diconjugate of bilirubin
11	$\bar{\alpha}_F$–$\bar{\beta}_x$	Related to bilirubin-IXβ and/or bilrubin-IXδ
12	$\bar{\alpha}_2$–$\bar{\alpha}_2$	Ester dixyloside of bilirubin-IXα
13	$\bar{\alpha}_2$–$\bar{\alpha}_3$	Monoxylose, monoglucose, diester of bilirubin-IXα
14	$\bar{\alpha}_3$–$\bar{\alpha}_3$	Ester diglucoside of bilirubin-IXα
15	($\bar{\alpha}_{0M}$–$\bar{\delta}$)	Presumably a monomethyl, monoglucuronic acid diester of bilirubin-IXα
16	($\bar{\gamma}$–$\bar{\gamma}$)	An ester diconjugate of bilirubin IXα
17	$\bar{\alpha}_0$–$\bar{\delta}$	Ester monoglucuronide of bilirubin-IXα
18	$\bar{\alpha}_2$–$\bar{\delta}$	Monoxylose, monoglucuronic acid diester of bilirubin-IXα
19	$\bar{\alpha}_3$–$\bar{\delta}$	Monoglucose, monoglucuronic acid diester of bilirubin-IXα
20	$\bar{\delta}$–$\bar{\delta}$	Ester diglucuronide of bilirubin-IXα

respectively (Table 1). To facilitate discussion diazo-positive bile pigments isolated by the present methods will be assumed to contain the biladiene-(a,c) skeleton, until the validity of this assumption can be evaluated. The pigments have been represented as follows. Greek letters with a bar above them represent the two dipyrroles present in the derivatives found after diazo-coupling. A bar between the letters denotes the central methylene bridge of the tetrapyrrole. For instance, bands yielding azopigment α_0, equimolecular mixtures of azopigments α_0 and α_2, or azopigment α_2 are depicted as $\bar{\alpha}_0$–$\bar{\alpha}_0$, $\bar{\alpha}_0$–$\bar{\alpha}_2$ and $\bar{\alpha}_2$–$\bar{\alpha}_2$ respectively (Table 2). Obviously, for the identification of these pigments as bilirubin, and its mono- and di-xyloside respectively, confirmatory structural evidence is needed.

Materials and Methods

Chemicals

Chemicals were usually of reagent grade. The chloroform used contained 0.6% ethanol as a stabilizer. Pentan-2-one (previously dried on $CaSO_4$) was redistilled before use. Aqueous solutions were prepared with twice-glass-distilled water. Bilirubin (quality: for biochemical purposes) was from E. Merck A.-G., Darmstadt, Germany. Two samples of [$^{14}C_8$]bilirubin, prepared biosynthetically from δ-amino[^{14}C]laevulinic acid (Ostrow & Murphy, 1970), were kindly provided by Dr. J. D. Ostrow (Veterans Administration Hospital, University of Pennsylvania Medical Service, Philadelphia, Pa. 19104, U.S.A.). After repurification (see below) the specific radioactivitivies were 2200 and 3400 d.p.s./μmol respectively.

Bile samples

Individual samples of gall-bladder bile, obtained from 45 dogs during surgery, were used. Without delay, samples (2ml) were delivered into glass-stoppered 10ml centrifuge tubes and were stored at about −15°C until further use. In some experiments pooled bile from 30 gall bladders was used (three pools were tested). To attain easy access to dog bile from a single dog a permanent catheter was placed in the gall bladder of one dog by the method of Cohen (1960). The experiment was only partially successful as the catheter became clogged at intervals. This produced increased amounts of so-called β- and γ-azopigments (Heirwegh *et al.*, 1970) that are typically found in cholestatic bile of man and rat (Fevery *et al.*, 1972*b*).

Methods

Except when stated otherwise, chromatography was at room temperature in the dark on glass plates pre-coated with silica gel (DC-Kieselgel F254, 5715/0025; E. Merck A.-G.). All centrifugations were at room temperature in an ordinary laboratory centrifuge at 3000rev./min ($1000g_{av.}$). The glycine–HCl buffer, frequently referred to below, was prepared by adjusting a 0.4M-HCl solution to pH 2.7 with solid glycine.

Extraction of bile pigments and separation by t.l.c. The manipulations were done with the minimum of delay. Solutions containing bile pigment were protected from light. The tubes were kept in contact with crushed ice when this was feasible.

Extraction procedure A. This was essentially the method of Folch *et al.* (1957). A portion (2ml) of frozen bile was thawed and mixed thoroughly with 7.5ml of cold methanol on a vortex mixer (Mixomat; Boskamp, Geräte Bau, Bonn, Germany). The supernatant solution obtained by centrifugation for 10min was transferred to a glass-stoppered centrifuge tube. Chloroform (15ml) and 3ml of a saturated solution of ascorbic acid were added and the mixture was shaken vigorously by hand and then centrifuged for 10min. The chloroform phase was removed and dried at about 15°C in a rotary evaporator *in vacuo*. The pigment residue was dissolved in 5ml of pentanone and further shaken with 10ml of water. The mixture was briefly centrifuged and parts of the upper organic phase (portions equivalent to about 0.5ml of bile) were applied as a streak (12cm) to thin-layer plates under evaporation in a stream of warm air.

Extraction procedure B. A sample (2ml) of frozen bile was thawed. Then 20ml of diluted solution containing 0.05–0.5μmol of total bilirubin/ml was shaken vigorously on the vortex mixer with 4g of ascorbic acid and 10ml of chloroform The organic phase was separated by brief centrifugation and further washed twice with equal volumes of water. Portions of the washed extract were applied as a streak (12cm) to thin-layer plates under evaporation in a stream of cold air.

The plates (procedures A and B) were developed with the following solvent systems: (1) two consecutive developments with chloroform–methanol–water (40:6:1, by vol.) (18cm), or (2) development with chloroform–methanol–water (60:35:8, by vol.) (3–6cm) followed by chloroform–methanol–water (40:9:1, by vol.) (18cm), or (3) a single development with chloroform–methanol–water (60:35:8, by vol.) (18cm). Plates were dried in an air stream between successive developments. The solvent contained the anti-oxidant, di-t-butyl-*p*-cresol (10mg/ml) (Wren & Szszepanowska, 1964). Separation of the more rapidly moving bile pigments was promoted by using solvent systems 1 or 2. A chromatogram is shown in Fig. 1. As a routine azo-derivatives were prepared immediately after separation (see below).

Formation and analysis of azopigments. Coloured bands were scraped from the plates and transferred to 10ml centrifuge tubes. The contents of each tube were shaken with 1ml of ethyl anthranilate diazo-reagent prepared as described by Heirwegh *et al.* (1970). The final pH value was about 1.4. The mixtures were left at room temperature. After 2.5–5min the coupling reaction was terminated by the addition of 5ml of freshly prepared ascorbic acid solution (50mg/ml). Note that no glycine–HCl buffer was added and that the reaction time (usually 30min) was decreased. The mixtures were shaken well, then centrifuged and the supernatant phases decanted. The sedimented silica-gel particles with the adsorbed azopigment(s) were mixed vigorously on the vortex mixer with 1ml of formamide to free the azopigment. The tubes were left at room temperature for about 30s and then mixed again; mixing was repeated 3–4 times at intervals of 30s. The contents of the tubes

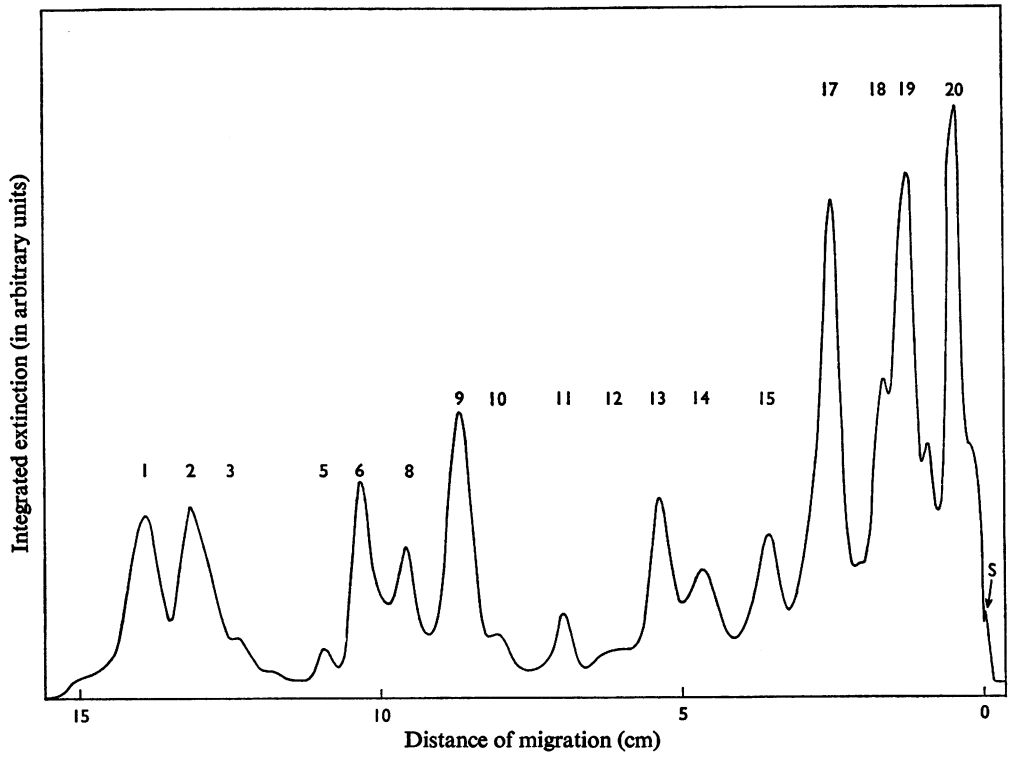


Fig. 1. *Densitometric tracing of preparative thin-layer chromatogram of bile pigments*

Pigments extracted from normal dog bile were separated by consecutive developments with chloroform–methanol–water (60:35:8, by vol.) (over 4cm) and with chloroform–methanol–water (40:9:1, by vol.) (over 16cm). The application line was situated at S. The significance of the reference numbers is given in Table 2. In the presently analysed bile sample component 9 ($\bar{\alpha}_0$–$\bar{\alpha}_3$) also yielded small amounts of the methyl ester of azodipyrrole, α_{OM}.

were further mixed with 1.5ml of pentanone. The supernatant solutions obtained by brief centrifugation were decanted and shaken with 8ml of glycine–HCl buffer, pH 2.7, to remove formamide. The organic phases were separated by brief centrifugation and submitted to t.l.c. with chloroform–methanol–water (65:25:3, by vol.) (over 5cm) followed by chloroform–methanol (17:3, v/v) (over 18cm). Preparations of purified azopigments (Table 1) or of total azopigment obtained from dog bile served as references.

The complete procedure, including extraction and separation of bile pigments, and colour formation and t.l.c. of the azo-derivatives, can be carried out in 1 day. If convenient, azopigment extracts can be stored in deep-freeze for several days before analysis. Some typical azopigment patterns are shown in Fig. 2.

Quantification of separated pigments. Relative amounts of bile pigments (measurement at 439nm) and azopigments (measurement at 536nm) were determined by densitometry (Fevery *et al.*, 1971). For recording bile pigment chromatograms either small spots (2cm) or a restricted area of a preparative chromatogram were scanned, as illumination at 439nm caused some fading of the coloured bands.

Vinyl–isovinyl isomer analysis of azodipyrrole. The method of Compernolle *et al.* (1970) was followed. Individual spots of separated azopigment α_0 obtained from bile pigment bands (see under 'Formation and analysis of azopigments') were scraped from thin-layer plates. The powders were transferred to small glass columns (internal diam. about 7mm) with elongated tips. Methanol eluates were prepared and submitted to t.l.c. with benzene–ethyl acetate (1:1, v/v) (two developments over 18cm). Azodipyrrole obtained from bilirubin served as a reference.

Methanolysis of conjugated azopigments and vinyl–isovinyl isomer analysis of the derived methyl ester of azodipyrrole. On treatment of purified preparations of conjugated azopigments α_2, α_3 and δ with NaOH

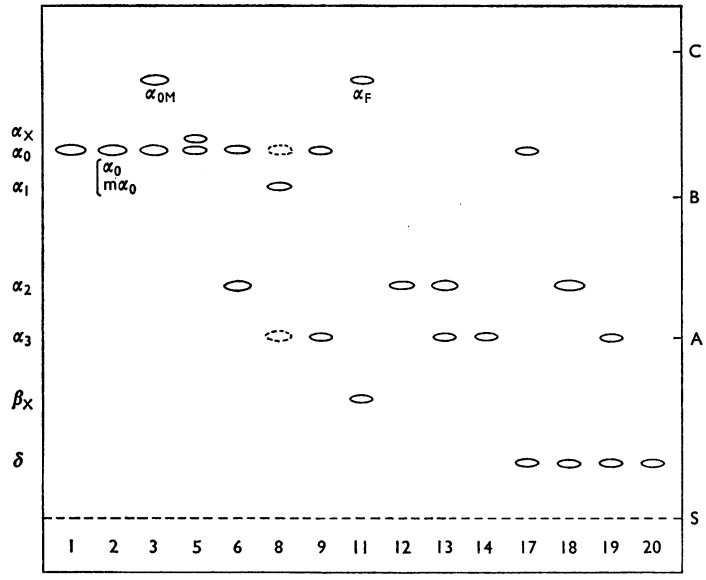


Fig. 2. *T.l.c. of azopigments obtained by treatment of isolated bile pigments with diazotized ethyl anthranilate*

The application line is indicated by S. Chromatograms were developed consecutively to levels A, B and C with chloroform–methanol–water (65:25:3, by vol.), chloroform–methanol (17:3, v/v) and chloroform–methanol (19:1, v/v) respectively. The relative distances of the spots from the origin are as observed in the actual separation. The reference numbers, indicated below the application line, denote the parent bile pigment bands (see Fig. 1 and Table 2). The significance of the Greek letter notation is explained in the text and in Table 1. From component 2 both azodipyrrole (α_0) and mesoazodipyrrole ($m\alpha_0$) were obtained.

in methanol the carboxylic acid methyl ester of azodipyrrole is formed. Methanol eluates of separated azopigments (see under 'Formation and analysis of azopigments') were mixed with an equal volume of NaOH (10mg/ml) in methanol. After 30–60s the content of each tube was neutralized with 3–8ml of glycine–HCl buffer, pH 2.7. After further thorough mixing with 0.5ml of chloroform the organic phases were separated by centrifugation and were submitted to t.l.c. with benzene–ethyl acetate (17:3, v/v) (over 18cm). The methyl ester of azodipyrrole served as a reference. The ratio between both isomers was determined by densitometry.

Ammonolysis of conjugated azopigments. Individual spots of separated conjugated azopigment (see under 'Formation and analysis of azopigments') were eluted from the powders with methanol. After repurification by t.l.c., methanol eluates of the azopigments were applied to a thin-layer plate. Treatment with NH_3 vapour and final analysis of the reaction products (the amide of azodipyrrole was accompanied, in general, by various amounts of the free acid) were made as described by Heirwegh *et al.* (1970).

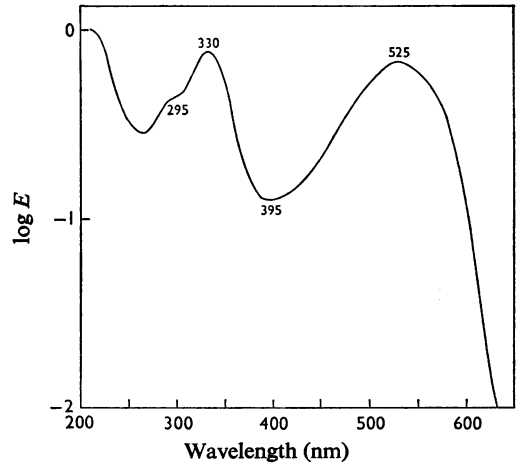


Fig. 3. *Characteristic absorption spectrum of azodipyrrole*

The absorption spectrum of purified azodipyrrole, dissolved in methanol, was obtained. Plotting of the logarithm of the extinction renders the shape of the curve independent of the concentration of the sample and of the optical path-length of the sample cuvette.

Determination of absorption spectra. Methanol eluates of separated azopigments (see under 'Formation and analysis of azopigments') were obtained. Preparations of azopigment α_0 were purified by t.l.c. with benzene–ethyl acetate (1:2, v/v) (over 18cm), repetitive development with chloroform–methanol (17:3, v/v) being used for purifying conjugated azopigments. The final methanol eluates were centrifuged for 10min. The absorption spectra of the supernatant solutions were recorded with a Beckman model 25 spectrophotometer (Fullerton, Calif. 92634, U.S.A.) at a scanning speed of 50nm/min. Characteristic spectra were obtained by plotting the logarithm of the extinction against the wavelength (Fig. 3). In this way comparison of spectra is facilitated as spectra of the same compound taken at different concentrations and/or optical pathlengths can be made to coincide by shifting along the log E axis, provided Beer's law is followed. For purified preparations of azodipyrrole and of azopigment α_3 Beer's law was followed from 220 to 700nm for solutions in methanol containing 0–43 μM-azopigment.

Formation of acetates of azopigments. The procedure of Compernolle *et al.* (1971) was adapted for micro work. Individual spots of separated azopigments (see under 'Formation and analysis of azopigments') were scraped from the plates. The powders were transferred to 10ml centrifuge tubes and treated for 1h at room temperature with 0.5ml of pyridine–acetic anhydride (2:1, v/v). Pentanone (1ml) was then added followed by seven successive 1ml portions of a saturated $NaHCO_3$ solution (waiting a few seconds after each addition). The reaction mixtures were left for 1h at room temperature, then mixed and briefly centrifuged. The organic phases were washed with 8ml of $NaHCO_3$ solution and after subsequent washing with glycine–HCl buffer, pH 2.7, the acetylated azopigments were separated by t.l.c. by successive developments with chloroform–methanol (17:3, v/v) (over 2cm) and benzene–ethyl acetate (17:3, v/v) (over 18cm). Acetates of azodipyrrole (β?)-D-xylopyranoside and of azodipyrrole β-D-glucopyranoside served as references (Table 1).

Treatment with diazomethane. Azopigment in pentanone–formamide (19:1, v/v) was treated with an equal volume of ethereal diazomethane. After 15–30s the excess of reagent was evaporated and the organic solution further washed with 10vol. of glycine–HCl buffer, pH 2.7. Extracted azodipyrrole methyl ester was developed on a thin-layer plate with benzene–ethyl acetate (17:3, v/v) (over 18cm) as the solvent. For the methyl ester of δ-azopigment chloroform–methanol (17:3, v/v) was used. Azodipyrrole, its methyl ester, and the methyl ester of azodipyrrole β-D-glucopyranuronic acid served as references (Table 1).

Alkaline hydrolysis of conjugated bilirubin and determination of IIIα, IXα and XIIIα structures. Ester glycosides rapidly hydrolyse at room temperature at pH 11.5 (Billing *et al.*, 1957). The present procedure is based on work of Ostrow *et al.* (1961), McDonagh & Assisi (1971) and Jansen (1973).

(*a*) Separated bile-pigment bands (see under 'Extraction of bile pigments and separation by t.l.c.') were scraped from the plates and treated in 10ml centrifuge tubes with 1ml of 1M-NaOH containing 10mg of ascorbic acid. The reaction mixtures were shaken at room temperature in the dark for 20min. The supernatants, obtained by brief centrifugation, were treated with 0.25ml of acetic acid (final pH about 4) and extracted immediately with 1ml of chloroform. The organic phases, separated by centrifugation, were applied to pre-coated silica-gel plates (Kieselgel 60, 5721/0205; E. Merck A.-G.) (Jansen, 1973) and developed with chloroform–acetic acid (99:1, v/v) (McDonagh & Assisi, 1971). Bilirubin served as a reference. The bilirubin isomers were quantified by densitometry. The pigments rapidly turned green during scanning.

(*b*) Samples (0.5ml) of gall-bladder bile of man and dog were treated in parallel. After the addition of 1ml of ethanol and 18.5ml of water, 5ml portions with 50mg of ascorbic acid added were further shaken in the dark at room temperature for 30min with 5ml of 1M-NaOH. The pH was adjusted to 5 with acetic acid and bile pigment was extracted with 20ml of chloroform. No bile pigment remained in the aqueous phase, conjugated bilirubin being absent from the chloroform extracts. They were analysed by t.l.c. and densitometry.

Incubation of dog bile with [$^{14}C_8$]bilirubin. Labelled bilirubin was stored as follows. The pigment was dissolved and divided into portions containing 250 μg of bilirubin. The solutions were evaporated to 1ml under N_2. Precipitation was obtained by further evaporation after the addition of 2ml of methanol (Black *et al.*, 1974). The preparations were stored dry in the dark at −15°C. Before each experiment a sample was repurified by preparative t.l.c. with chloroform as the solvent. Two preparations with specific radioactivities of 2000 and 3400d.p.s./μmol respectively were used in the experiments described below.

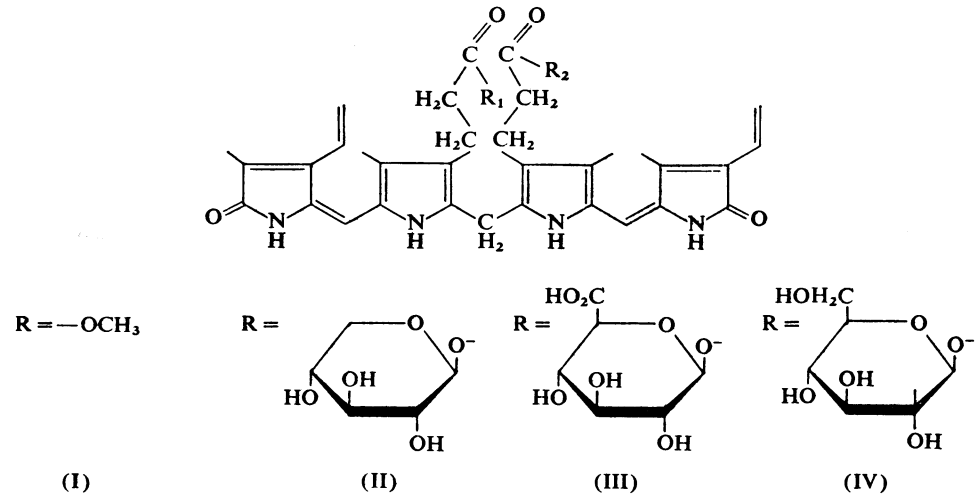
The pigment was eluted from the powder with chloroform and evaporated to dryness. The residue was rapidly dissolved in 0.2ml of 0.15M-NaOH and treated immediately with 2ml of phosphate buffer, pH 7.4 (17.6ml of 0.5M-KH_2PO_4 and 60.8ml of 0.5M-Na_2HPO_4 diluted to 1000ml) and 0.5ml of dog bile (final pH 7.7). A similar mixture, containing no labelled bilirubin, served as a control. Both mixtures were placed in a Dubnov shaking incubator in the dark at 37°C for 30min. Bile pigment was then extracted by procedure A. The chloroform phase was

evaporated to dryness and the residue redissolved in 5ml of pentanone. The solution was washed with 10ml of ascorbic acid solution (50mg/ml) instead of water. The pentanone extract (2ml) was submitted to t.l.c. with chloroform–methanol–water (40:9:1, by vol.) as the solvent (development over 18cm). The bile-pigment bands were scraped from the plates and were treated with diazotized ethyl anthranilate. The azo-derivatives were separated by t.l.c. by successive development with chloroform–methanol (17:3, v/v) (over 10cm) and benzene–ethyl acetate (3:2, v/v) (over 18cm). The fractions of azopigment α_0 obtained from bile-pigment bands 1 ($\bar{\alpha}_0$–$\bar{\alpha}_0$), 5 ($\bar{\alpha}_0$–$\bar{\alpha}_x$), 7 ($\bar{\alpha}_0$–$\bar{\alpha}_2$), 9 ($\bar{\alpha}_0$–$\bar{\alpha}_3$) and 17 ($\bar{\alpha}_0$–$\bar{\delta}$) were eluted separately with pentanone–formamide (9:1, v/v) and were submitted to further treatment with diazomethane. On subsequent t.l.c. with benzene–ethyl acetate (9:1, v/v) (over 18cm) the derivatives separated each into two spots moving as the vinyl and isovinyl isomers of azodipyrrole methyl ester respectively. Each pair of derivatives was combined and eluted with methanol and then rechromatographed with benzene–ethyl acetate (3:2, v/v) (over 18cm). The methyl esters were finally eluted from the powders with 1ml of acetone–water (9:1, v/v) and used subsequently for photometric determination at 546nm and for radioactivity counting. To 1ml of the acetone–water solution 9ml of Instagel (Packard Instrument Co. Inc., Warrenville, Ill., U.S.A.) was added. The samples were counted for 10min in a Packard Tri-Carb liquid-scintillation spectrometer, model 2450 (Packard Instrument Co. Inc.).

Results

The present t.l.c. method designed for direct separation of conjugated bile pigments yielded a number of yellow-to-brownish bands (Fig. 1) that reacted with diazotized ethyl anthranilate to form reddish-violet azo-derivatives (Fig. 2). The formation of dipyrrolic azopigments (see below) is compatible with a variety of (*a*) biladiene-(a,c) structures such as bilirubin IXα or IXβ, and (*b*) bilene-a or -c structures (Görges & Gohr, 1954; N. Blanckaert, unpublished work). Evidence given below indicates that except for components 2 and 11 (Table 2), the pigments either correspond to unconjugated (band-1) or to conjugated bilirubin-IXα (Scheme 1).

Structure elucidation of conjugated tetrapyrroles requires information about (*a*) the nature and isomeric composition of the aglycone, (*b*) the number of the conjugating groups present per molecule of aglycone and their nature, and (*c*) the nature of the



Scheme 1. *Structures of bilirubin-IXα conjugates detected in dog bile*

From left to right conjugating groups (R) represent (I) methoxyl, (II) β-D-xylosyl, (III) β-D-glucuronosyl and (IV) β-D-glucosyl residues. Bilirubin is obtained when $R^1 = R^2 = OH$; monoconjugates have either $R^1 = OH$ or $R^2 = OH$, the conjugating groups being equal to I ($\bar{\alpha}_0$–$\bar{\alpha}_{0M}$), II ($\bar{\alpha}_0$–$\bar{\alpha}_2$), III ($\bar{\alpha}_0$–$\bar{\delta}$) or IV ($\bar{\alpha}_0$–$\bar{\alpha}_3$); homogeneous diconjugates R^1 and R^2 equal to II ($\bar{\alpha}_2$–$\bar{\alpha}_2$), III ($\bar{\delta}$–$\bar{\delta}$) or IV ($\bar{\alpha}_3$–$\bar{\alpha}_3$); mixed diconjugates are combinations of $R^1 = II$ and $R^2 = IV$ ($\bar{\alpha}_2$–$\bar{\alpha}_3$), and $R^1 =$ III and R^2 equal to I ($\bar{\alpha}_{0M}$–$\bar{\delta}$), II ($\bar{\alpha}_2$–$\bar{\delta}$) or IV ($\bar{\alpha}_3$–$\bar{\delta}$). The conjugating structures of some pigments are incompletely known: $\bar{\alpha}_0$–$\bar{\alpha}_1$, $\bar{\alpha}_0$–$\bar{\alpha}_x$, $\bar{\alpha}_x$–$\bar{\alpha}_x$, $\bar{\alpha}_1$–$\bar{\alpha}_1$, $\bar{\alpha}_3'$–$\bar{\alpha}_3'$ and $\bar{\gamma}$–$\bar{\gamma}$.

bonds connecting conjugating groups to aglycone. As, in general, mono- and mixed di-conjugates of the asymmetrical aglycone, bilirubin-IXα, can be present in bile, the possibility (*d*) of positional isomerism must also be investigated. For the present, only point (*a*) can be investigated at the stage of the tetrapyrroles. Evidence with regard to points (*b*)–(*d*) derives largely from analysing dipyrrolic azo derivatives.

After presenting the method for extraction and separation of diazo-positive bile pigments, the information obtained by azopigment analysis will be discussed with regard to the structure of the parent tetrapyrroles. To facilitate the discussion a numbered list of diazo-positive bands together with a summary of structural information has been compiled (Table 2).

Extraction and separation of bile pigments

In attempts to achieve quantitative extraction several procedures were tested. Initial experiments with pentanone containing 0–10% (v/v) butan-1-ol indicated that previous acidification of bile with ascorbic acid to about pH 3 considerably improved yields of pigments. The effect is probably related to decreasing ionization of carboxyl groups. T.l.c. of these extracts, however, demonstrated irregular shapes of the pigment bands owing to the presence of butanol. Therefore procedures A and B were tested.

The extraction yields were estimated on the assumption that the molecular extinction coefficients at 436nm of bile pigments in diluted bile, and in methanol or chloroform, are comparable. Bile samples from six dogs were treated by procedure A (1.9–5.0μmol of bile pigment/assay). Of the initial colour, 76–100% (average 89%) was recovered in the methanol supernatants but only 33–67% (average 44%) was found in the final chloroform solutions. With procedure B recovery in the chloroform extracts amounted to 73, 70, 67 and 67%, when dilutions from a single bile sample, containing respectively 1.0, 2.0, 5.1 and 10.1μmol of bile pigment were tested (pH of the aqueous phase: 2.1–2.5). On repetition of the experiment with three individual bile samples containing 3.2, 4.6 and 9.4μmol of bile pigment respectively, the extraction yields were 65, 51 and 67%.

Procedure B shows somewhat better yields and is less complex than procedure A. It was used in the studies reported below except when stated otherwise. However, occasionally poor separation was obtained. This behaviour appeared to be typical for certain bile specimens, as no improvement was obtained on repeated trials. Evaporation of the washed chloroform extract *in vacuo* followed by solution of the residue in pentanone and washing with water (as in procedure A), in most instances, improved chromatographic behaviour. In a typical experiment resolution of pigment bands, judged by visual inspection of the plates, was adequate when 0.012–0.060μmol of bile pigment was applied/1cm length of the application line. A subsequent check on the same bile specimen with procedure A demonstrated that resolution was at least as good. Obviously in its modified form procedure B offers no clear-cut advantage. Initially, another reason to reject precedure A was the presence of large amounts of methanol in the extracts. Conceivably, this could induce some methylation of carboxyl groups or methanolysis of ester conjugates. According to our present experience, however, this is of little if any importance (see below).

As a routine yellow-to-brownish bands were selected for further analysis by visual inspection of the chromatograms. This procedure entails the risk of overlooking minor compounds. In six experiments a densitometric record was prepared to aid localization (see e.g. Fig. 1); bands corresponding to regions around the maxima of the densitometric tracings were submitted to diazo-coupling. Both methods yielded essentially the same sort of information. The pigment bands shown in Fig. 1, except for components 3 ($\bar{\alpha}_0$–$\bar{\alpha}_{0M}$), 10 ($\bar{\alpha}_3'$–$\bar{\alpha}_3'$) and 15 ($\bar{\alpha}_{0M}$–$\bar{\delta}$), were detected in all bile samples analysed (50 analyses; 40 bile samples) (Table 2). Band 2, characterized by yielding both azodipyrrole and meso-azodipyrrole (N. Blanckaert, unpublished work) occasionally separated into two components with apparently the same compositions. Component 3 was followed by one or two faintly yellow diazo-positive bands. Undoubtedly, the list given in Table 2 is not exhaustive. Components 4 ($\bar{\alpha}_0$–$\bar{\alpha}_1$), 6 ($\bar{\alpha}_x$–$\bar{\alpha}_x$), 10 ($\bar{\alpha}_3'$–$\bar{\alpha}_3'$), 15 ($\bar{\alpha}_{0M}$–$\bar{\delta}$) and 16 ($\bar{\gamma}$–$\bar{\gamma}$) were observed only four, four, four, two and five times respectively. In about 25% of the analyses the δ-azopigment from bands 17 ($\bar{\alpha}_0$–$\bar{\delta}$) and 20 ($\bar{\delta}$–$\bar{\delta}$) was still accompanied by some γ-azopigment. Component 11 ($\bar{\alpha}_F$–$\bar{\beta}_x$) was not detected in two bile samples.

To aid recognition of chromatographic bands several observations can be mentioned. The relative chromatographic mobilities can be appreciated from Fig. 1 and Table 2. In general, pigments 9 ($\bar{\alpha}_0$–$\bar{\alpha}_3$) and 17 ($\bar{\alpha}_0$–$\bar{\delta}$) were predominant among the better resolved components. Frequently, component 11 ($\bar{\alpha}_F$–$\bar{\beta}_x$) was closely followed by a greyish band whereas component 13 ($\bar{\alpha}_2$–$\bar{\alpha}_3$) was immediately preceded by a red band and was followed by an orange band. Reddish-brown material appeared gradually in the region between components 2 and 5 when chromatographic development was stopped. However, rapid and unequivocal identification can conveniently be done by azopigment analysis (Fig. 2).

Analysis and structure determination of azo-derivatives

Pigment 11 ($\bar{\alpha}_F$–$\bar{\beta}_x$) has been mentioned for the sake of completeness. The available evidence indicates that it is a biladiene-(a,c) with a skeleton other than bilirubin-IXα (N. Blanckaert, unpublished work). Mesodihydrobilirubin, or material closely related to it, was found between bands 1 and 3 (N. Blanckaert, unpublished work). These components will further be disregarded, thus making possible a more simple and unified discussion of the remaining pigments.

Formation and t.l.c. of the azo-derivatives. Except for component 1 ($\bar{\alpha}_0$–$\bar{\alpha}_0$) azo-colour formation was nearly instantaneous and appeared to be complete. With pigments 12–14 and 18–20 treatment with diazo-reagent for 15–60min produced progressively increasing but still relatively small amounts of azopigment α_0. Partial hydrolysis in the acid reaction medium is suggested. Release of azopigment from the silica-gel particles by treatment with formamide is a time-dependent process promoted by intermittent mixing of the suspensions. Eventually, practically all azopigment was recovered in the supernatant fluids. Successful t.l.c. of the azo-derivatives requires previous removal of formamide.

The azopigment patterns obtained from the bile pigment bands are shown in Fig. 2. Essentially similar results were found with extraction procedures A and B and with extraction solvent composed of pentanone containing 0–10% (v/v) butan-1-ol. Details (see below) refer to procedure B. From components 1, 5–10, 12–14, 16 and 17 either one predominant azopigment (Table 3) or mixtures of two azopigments, in a colour ratio near to 1 (Table 4), were derived. In general, accompanying azopigment(s) did not exceed 5% of total azo-colour except for band 8 ($\bar{\alpha}_1$–$\bar{\alpha}_1$). In this case, azopigment α_1 amounted to 60–85% of total colour (six analyses), remaining colour being about equally distributed over azopigments α_0 and α_3; azopigment α_2 occurred in trace amounts.

Visual inspection of numerous other separations amply confirmed the previous conclusions. In general, the uniqueness of the azopigment compositions was striking. The compositions that are implied in formulae ($\bar{\alpha}_0$–$\bar{\alpha}_{0M}$), ($\bar{\alpha}_0$–$\bar{\alpha}_1$) and ($\bar{\alpha}_{0M}$–$\bar{\delta}$) of bands 3, 4 and 15, respectively, are somewhat less certain, as they are not based on densitometry but only on visual inspection of chromatograms. Azopigments α_x and α_1 are poorly distinguished from azopigment α_0 in the routine solvent system, chloroform–methanol–water (65:25:3, by vol.). Some improvement is obtained by subsequent development with chloroform–methanol (17:3, v/v) (Fig. 2). They do separate in the solvent systems mentioned in Table 5.

In the region occupied by the more hydrophilic components 18–20, formation of one or two predominant equally important derivatives was also observed (Tables 3 and 4). However, the relative amounts of accompanying azopigments were frequently larger than was found with the more rapidly moving bands, indicating more pronounced overlapping of the tetrapyrroles (Fig. 1). In 8 of 16 analyses with solvent system 3 splitting of bands 17 ($\bar{\alpha}_0$–$\bar{\delta}$) and/or 19 ($\bar{\alpha}_3$–$\bar{\delta}$) and/or 20 ($\bar{\delta}$–$\bar{\delta}$) was observed. In total, this multiplicity was found four times for each type of pigment.

Table 3. *Vinyl–isovinyl isomer composition of azopigments derived from bilirubin and homogeneous diconjugates*

For bilirubin the t.l.c. analyses were performed on the α_0-azopigment. For the conjugates methyl ester azopigments obtained by alkaline methanolysis from separated azopigments were analysed by t.l.c. If the bile pigments contain the bilirubin-IXα nucleus equal amounts of the dipyrrolic vinyl and isovinyl isomers should be found. The same bile samples were analysed as mentioned in Table 4.

Experiment no.	Bile pigment band		% of vinyl isomer (theoretical value = 50)
	No.	Formula	
1	1	$\bar{\alpha}_0$–$\bar{\alpha}_0$	51
	8	$\bar{\alpha}_1$–$\bar{\alpha}_1$	50
	14	$\bar{\alpha}_3$–$\bar{\alpha}_3$	40
2	8	$\bar{\alpha}_1$–$\bar{\alpha}_1$	50
	12	$\bar{\alpha}_2$–$\bar{\alpha}_2$	57
	14	$\bar{\alpha}_3$–$\bar{\alpha}_3$	54
	16	$\bar{\gamma}$–$\bar{\gamma}$	53
	20	$\bar{\delta}$–$\bar{\delta}$	54
3	8	$\bar{\alpha}_1$–$\bar{\alpha}_1$	49
	12	$\bar{\alpha}_2$–$\bar{\alpha}_2$	51
	14	$\bar{\alpha}_3$–$\bar{\alpha}_3$	50
	20	$\bar{\delta}$–$\bar{\delta}$	54
4	8	$\bar{\alpha}_1$–$\bar{\alpha}_1$	45
	12	$\bar{\alpha}_2$–$\bar{\alpha}_2$	54
	14	$\bar{\alpha}_3$–$\bar{\alpha}_3$	49

Vinyl–isovinyl isomer analysis of α_0-azopigment derived from separated bile pigments. T.l.c. of the α_0-azopigment obtained from bands 1 ($\bar{\alpha}_0$–$\bar{\alpha}_0$), 7 ($\bar{\alpha}_0$–$\bar{\alpha}_2$), 9 ($\bar{\alpha}_0$–$\bar{\alpha}_3$) and 17 ($\bar{\alpha}_0$–$\bar{\delta}$) in each case yielded components moving as the vinyl and isovinyl isomers of authentic azodipyrrole respectively. After treatment with diazomethane the azopigments yielded two methyl esters moving chromatographically as the vinyl and isovinyl isomers of authentic azodipyrrole methyl ester (six bile samples analysed with both methods). Both separation as the free acid and as the methyl ester yielded the same vinyl/isovinyl ratios (Table 6) supporting the validity of the procedures. In general, roughly comparable amounts of both isomers were measured (Tables 3, 4 and 6). The α_0-azopigment of band 5 ($\bar{\alpha}_0$–$\bar{\alpha}_x$) showed similar resolution into two isomers for four bile specimens, but

Table 4. *Azopigment and vinyl–isovinyl isomer composition of separated monoconjugates and mixed diconjugates of bilirubin*

For the unconjugated halves of monoconjugates of bilirubin the vinyl–isovinyl isomer determination was done on the α_0-azopigment. T.l.c. of methyl ester azopigments obtained by alkaline methanolysis was used for the conjugated azopigments. The letters a and b refer to the azopigments from the dipyrrole halves written at the left-hand side and right-hand side of the formulae respectively. Theoretically, either azo-derivative should represent 50% of total azo-colour. The percentages of vinyl isomer (V) of azopigment-a and of isovinyl isomer (I) of azopigment-b should be equal if the bile pigments contain the bilirubin-IXα nucleus. The same bile samples are analysed as are mentioned in Table 3.

Experiment no. / Azopigment ...	Bile pigment band No.	a b	% of azopigment-a (theoretical value = 50)	% of isomer: Vinyl (a_V) (theoretical: $a_V = b_I$)	% of isomer: Isovinyl (b_I)
1	5	$\bar{\alpha}_0$–$\bar{\alpha}_x$	49	51	46
	7	$\bar{\alpha}_0$–$\bar{\alpha}_2$	50	49	53
	9	$\bar{\alpha}_0$–$\bar{\alpha}_3$	49	50	55
	13	$\bar{\alpha}_2$–$\bar{\alpha}_3$	46	53	53
	17	$\bar{\alpha}_0$–$\bar{\delta}$	51	45	42
2	7	$\bar{\alpha}_0$–$\bar{\alpha}_2$	50	53	50
	9	$\bar{\alpha}_0$–$\bar{\alpha}_3$	49	59	56
	13	$\bar{\alpha}_2$–$\bar{\alpha}_3$	54	57	47
	17	$\bar{\alpha}_0$–$\bar{\delta}$	50	43	38
	18	$\bar{\alpha}_2$–$\bar{\delta}$	50	56	61
	19	$\bar{\alpha}_3$–$\bar{\delta}$	48	52	62
3	5	$\bar{\alpha}_0$–$\bar{\alpha}_x$	48	47	59
	7	$\bar{\alpha}_0$–$\bar{\alpha}_2$	53	58	50
	9	$\bar{\alpha}_0$–$\bar{\alpha}_3$	50	60	53
	13	$\bar{\alpha}_2$–$\bar{\alpha}_3$	48	54	50
	17	$\bar{\alpha}_0$–$\bar{\delta}$	43	51	42
	18	$\bar{\alpha}_2$–$\bar{\delta}$	47	43	42
	19	$\bar{\alpha}_3$–$\bar{\delta}$	49	48	36
4	5	$\bar{\alpha}_0$–$\bar{\alpha}_x$		50	53
	7	$\bar{\alpha}_0$–$\bar{\alpha}_2$		56	55
	9	$\bar{\alpha}_0$–$\bar{\alpha}_3$		57	56
	13	$\bar{\alpha}_2$–$\bar{\alpha}_3$		52	53
	17	$\bar{\alpha}_0$–$\bar{\delta}$		52	41
	18	$\bar{\alpha}_2$–$\bar{\delta}$		43	41
	19	$\bar{\alpha}_3$–$\bar{\delta}$		47	39

Table 5. *$R_{azodipyrrole}$ values of some dipyrrolic azopigments*

The following developing solvents were used: (1) chloroform–methanol (17:3, v/v) (two developments over 2cm) followed by chloroform–methanol (19:1, v/v) (18cm); (2) chloroform–methanol (19:1, v/v) (over 18cm); (3) chloroform–methanol (17:3, v/v) (two developments over 2cm) followed by benzene–ethyl acetate (17:3, v/v) (two developments over 18cm); (4) benzene–ethyl acetate (3:2, v/v) (over 18cm). Plates were dried in an air stream between successive developments. Further details are given in the Materials and Methods Section.

Compound / Solvent ...	$R_{azodipyrrole}$ (1)	(2)	(3)	(4)
Azodipyrrole	1.00	1.00	1.00	1.00
Azodipyrrole amide (α_{0N})	1.12	1.30	0.61	0.71
Azopigment α_x	0.97	1.12	0.51	0.85
Azopigment α_1	0.38		0.25	
Azopigment α_2	0.25		0.16	
Azopigment α_3	0.09		0.00	

Table 6. *Vinyl–isovinyl isomer composition of azodipyrrole (α_0), obtained from separated bile pigment, and of the methyl ester derivative (α_{0M}) obtained by treatment of azopigment α_0 with diazomethane*

For details see the text.

Bile pigment band No.	Formula	% of vinyl isomer as azopigment	
1	$\bar{\alpha}_0$–$\bar{\alpha}_0$	56	57
5	$\bar{\alpha}_0$–$\bar{\alpha}_x$	100	100
7	$\bar{\alpha}_0$–$\bar{\alpha}_2$	60	61
9	$\bar{\alpha}_0$–$\bar{\alpha}_3$	62	60
17	$\bar{\alpha}_0$–$\bar{\delta}$	62	59

in two other cases only the vinyl isomer was obtained.

Mesoazodipyrrole is not separated as the free acid from azodipyrrole, but separation occurs after

methylation (N. Blanckaert, unpublished work). The methyl ester analyses mentioned above showed the absence of the meso compound from the azodipyrrole preparations.

Ammonolysis of conjugated azopigments. Treatment of the azopigments α_x, α_1, α_2, α_3 and δ with NH_3 vapour (one bile sample analysed) yielded azodipyrrole amide and variable amounts of azodipyrrole indicating that groups bound in ester-linkage to the carboxyl groups of azodipyrrole are present in the conjugated azopigments. In another experiment the δ-preparations from bands 17 ($\bar{\alpha}_0$–$\bar{\delta}$), 18 ($\bar{\alpha}_2$–$\bar{\delta}$), 19 ($\bar{\alpha}_3$–$\bar{\delta}$) and 20 ($\bar{\delta}$–$\bar{\delta}$) were analysed. The δ-azopigment from the latter bile pigment showed partial resistance to the treatment whereas the other preparations were converted completely into mixtures of the free acid and the amide of azodipyrrole. This observation suggests that a mono- or di-conjugated alkali-stable bile pigment may have been present in band 20.

Methanolysis of conjugated azopigments; vinyl–isovinyl isomer analysis of the derived azodipyrrole methyl ester. The micro procedure was tested with purified preparations of azopigments α_2, α_3 and δ from dog bile. Conversion into the azodipyrrole methyl ester was complete after 30–60s in methanol solution containing NaOH (5 mg/ml). Prolonged treatment (30s to 5min) produced increasing amounts of azo-derivative moving somewhat more slowly than the less mobile vinyl isomer; occasionally, reddish material moving between the two main bands was also found. With a preparation of δ-azopigment 20s treatment produced complete conversion (vinyl/isovinyl isomer ratio = 0.68) with less than 5% of unidentified reaction products. Longer treatment for 1, 3 and 5min produced ratios of 0.68, 0.71 and 0.72 respectively, with progressively increasing amounts of unidentified material. More rapid destruction of the isovinyl isomer is suggested (a greater thermal degradation was observed for isovinyl isomers by mass spectrometry). Comparable ratios were found when a δ-preparation was treated for 35s at final NaOH concentrations of 2, 4, 6, 8 and 10mg/ml; the conversion was incomplete at the two lower concentrations.

The analyses yield much useful information. Methanolysis of conjugated azodipyrroles provides a simple and rapid test for the presence of conjugating groups bound in ester linkage to azodipyrrole. The results obtained by ammonolysis, although of similar nature, are less clear-cut as invariably the formation of the amide of azodipyrrole is accompanied by production of the free acid. Vinyl–isovinyl isomer analysis of methyl ester derivatives formed by methanolysis is easily performed and allows confirmation of the isomeric structure of the aglycone of the tetrapyrrolic conjugate. Two cases are of interest. (*a*) Homogeneous diconjugates of bilirubin-IXα should yield equal amounts of vinyl and isovinyl isomers of azodipyrrole methyl ester. Bilirubin-IXα similarly yields the vinyl and isovinyl isomers of azodipyrrole (Table 3). (*b*) For monoconjugates and mixed diconjugates of bilirubin-IXα a useful relationship can be deduced as follows. If dipyrrole halves and b are present in the tetrapyrrole a–b then combinations a_V–b_I and a_I–b_V are possible, where the subscripts V and I indicate dipyrrole halves with the vinyl and isovinyl isomeric structure respectively. On diazo-treatment and separation the azopigments a and b, each containing both isomers, are obtained. Obviously, the percentage of vinyl isomer for azopigment-a should equal the percentage of isovinyl isomer for azopigment-b (Table 4). The relationships remain valid when the aglycone of the conjugated tetrapyrrole contains either equimolecular mixtures of bilirubin-IIIα and -XIIIα or the said mixture plus some amount of bilirubin-IXα. To distinguish between these possibilities independent determination of the structure of the aglycone at the tetrapyrrolic stage is needed. The expected relationships were verified reasonably closely (Tables 3 and 4). Conversely, isomer determination at the dipyrrolic stage allows allocation of the conjugating groups to specific dipyrrole halves of the parent tetrapyrrole; as yet this cannot be done at the tetrapyrrolic stage.

Detection of mesoazodipyrrole methyl ester is obscured by possible formation of side products that move with about the same R_F values. Under conditions similar to those applied to effect methanolysis a variety of other alkyl esters can be formed. Conversion slows down with increasing chain length. Ethanolysis in alkaline medium apparently yielded exclusively the ethyl esters of azodipyrrole and thus may be more suitable to detect mesoazodipyrrole (N. Blanckaert, unpublished work). Lack of formation of side products with the latter procedure in contrast with alkaline methanolysis is compatible with a recent observation of Gordon *et al.* (1974). Side products obtained on methanolysis of azopigment α_3 with sodium methoxide were interpreted by the latter authors to result from replacement of the ethyl ester group of the anthranilate moiety by a methyl group.

Acetylation and methylation of conjugated azopigments. Extracts from pooled dog bile were separated by t.l.c. On acetylation of azopigments α_2 and α_3, derived from components 7, 9, 12–14, 18 and 19, pairs of derivatives were formed that moved chromatographically as the vinyl and isovinyl isomer pairs of azodipyrrole (β?)-D-xylopyranoside and azodipyrrole β-D-glucopyranoside respectively. Azopigments α_x and α_1 reacted similarly with acetylating agent forming derivatives more hydrophobic than the parent compounds (Tables 5 and 7). However, no splitting into two closely moving components was

Table 7. *$R_{azodipyrrole}$ values of acetate derivatives of conjugated azopigments*

Chromatographic development was with chloroform–methanol (17:3, v/v) (over 2cm) followed by benzene–ethyl acetate (17:3, v/v) (over 18cm). Further details are given in the Materials and Methods Section.

Compound	$R_{azodipyrrole}$ value
Acetate of azopigment α_X	5.43
Acetate of azopigment α_2	
Isovinyl isomer	5.18
Vinyl isomer	4.71
Acetate of azopigment α_1	4.10
Acetate of azopigment α_3	
Isovinyl isomer	3.71
Vinyl isomer	3.00
Azodipyrrole	1.00

achieved. It should be noted that parallel methanolysis assays demonstrated the presence of both the vinyl and isovinyl dipyrrole halves. The observations with regard to azopigments α_x, α_1, α_2 and α_3 were confirmed when, in addition, four individual bile samples were separated and analysed.

Both azopigments α_x and α_1 were stable at rechromatography. No structurally known references are available. Azopigment α_1, detected previously by azopigment analysis of whole dog bile (Fevery *et al.*, 1971), was prepared from dog bile and was purified by preparative t.l.c. (Table 5). The purified pigment and α_1-azopigment obtained from band 8 ($\bar{\alpha}_1$–$\bar{\alpha}_1$) showed identical chromatographic behaviour, both as the untreated (Table 5) and as the acetylated materials (Table 7). The $R_{azodipyrrole}$ values of azopigment α_x and of the amide of azodipyrrole showed remarkable inversion of R_F values in solvent systems 3 and 4 mentioned in Table 5. However, in parallel assays with acetic anhydride no acetylation occurred with the amide in contrast with α_x (Table 7).

The R_F values of azopigments α_x, α_1, α_2 and α_3 did not change on treatment with diazomethane. Azopigment δ, derived from bands 17–20 (two bile samples analysed) yielded one major derivative moving as the methyl ester of synthetic azodipyrrole β-D-glucopyranuronic acid (Table 1).

Absorption spectra of azo-derivatives. The absorption spectra of azopigments α_x, α_0, α_1, α_2, α_3 and δ, derived from isolated bile pigments, typically showed maxima at 330 and 528 nm, minima at 265 and 395 nm and a shoulder around 295 nm. They were approximately superimposable from 305 to 650 nm. The spectrum of purified azodipyrrole is shown in Fig. 3.

No contamination with the mesoazodipyrrole chromophore was detected, thus complementing the methylation tests that demonstrated absence of contamination only from the α_0-preparations. The presence of the mesochromophore would have been revealed not only by a shift of the absorption maximum to shorter wavelengths (Kuenzle, 1970*a*) but more sensitively by similar shifts of the ascending and descending sides of the whole absorption band (N. Blanckaert, unpublished work).

Apparent formation of the methyl ester of azodipyrrole by diazo-coupling of separated bile pigments. Occasionally, band 9 ($\bar{\alpha}_0$–$\bar{\alpha}_3$) yielded not only azopigments α_0 and α_3, but also small amounts of azopigment (presumably $\bar{\alpha}_{0M}$) moving with the solvent front in the routine chromatographic system (Fig. 2). Pigments with similar chromatographic behaviour were obtained from bands 3 ($\bar{\alpha}_0$–$\bar{\alpha}_{0M}$) and 15 ($\bar{\alpha}_{0M}$–δ). On one occasion they were isolated and rechromatographed with benzene–ethyl acetate (9:1, v/v) (over 18cm) as the solvent, with the methyl ester of azodipyrrole as a reference. A component moving as the vinyl isomer of the reference compound predominated with smaller amounts of apparent isovinyl isomer and of a more rapidly moving component. In the early stages of the present work this phenomenon was given little attention as (*a*) the amounts of pigments were rather small, (*b*) they did not appear in all bile samples and (*c*) were thought to be an artifact generated by the presence of relatively large amounts of methanol in the initial extracts (procedure A). However, it became apparent that the derivatives were observed with some bile samples, not with others: they were generated from several bands by using either procedure A or B. Also, addition of methanol to isolated bile pigment bands on silica gel and treatment with diazo-reagent did not cause formation of the pigments. Further, it may be noted that occasionally direct treatment of bile samples with diazo-reagent in the absence of methanol gave rise to the formation of azodipyrrole methyl ester. It thus seems likely that a group of bile pigments containing a methyl ester conjugating group may be present in dog bile.

Direct tests on tetrapyrrolic structure

Although much information can be derived from vinyl–isovinyl isomer analysis of dipyrrolic azo-derivatives such measurements are to some extent ambiguous. Indeed, bilirubin-IXα or equimolecular mixtures of the IIIα and XIIIα isomers both yield equal amounts of vinyl and isovinyl isomers of azodipyrrole. Although it is very likely that in the chromatographic systems used in the present work the IXα isomer is easily distinguished from the IXβ and IXδ isomers (N. Blanckaert, unpublished work), nothing is known about the relative chromatographic mobility of bilirubin-IXγ. Suitable micro modification of the procedure of O'Carra & Coleran (1970) for analysing bilirubin isomers IXα, IXβ, IXγ and IXδ as dimethyl esters of biliverdin would be useful. Another problem relates to differentiation between biladiene-(a,c) and bilene-a or -c tetrapyrroles, that all could yield azodipyrrole on diazo-coupling.

Finally, in determining structures of isolated tetrapyrroles possible isomerization by exchange of dipyrrole moieties preceding isolation should be taken into consideration (Stoll & Gray, 1970). Under certain conditions including photo-catalysis such rearrangement may occur in aqueous solutions of bilirubin (McDonagh & Assisi, 1972) and of its monoglucuronide (Jansen, 1973).

Determination of the IIIα, IXα *and* XIIIα *contents of reference bilirubin and of total and isolated bile pigments.* Commercial bilirubin and bilirubin obtained by alkaline hydrolysis from bile of man and dog were composed predominantly of bilirubin-IXα. The total amount of IIIα and XIIIα was about 6%. The analysis, repeated for the separated bile pigments, yielded a similar result. The same conclusion has been reached by others for bile of man (Kuenzle, 1970*a*; McDonagh & Assisi, 1972) and rats (McDonagh & Assisi, 1972).

Specific activity of azodipyrrole methyl ester obtained from separated bile pigment bands after preincubation of dog bile with [$^{14}C_8$]*bilirubin.* To investigate possible exchange of dipyrrole moieties small amounts of [$^{14}C_8$]bilirubin were incubated for 30min at 37°C with dog bile. Bilirubin pigments were separated and examined for radioactivity. The efficiency of radioactivity counting was between 59 and 63% of the theoretical value. After correction for background counts the specific radioactivities of the methyl ester of azodipyrrole derived from bands 1 ($\bar{\alpha}_0$–$\bar{\alpha}_0$), 7 ($\bar{\alpha}_0$–$\bar{\alpha}_2$), 9 ($\bar{\alpha}_0$–$\bar{\alpha}_3$) and 17 ($\bar{\alpha}_0$–$\bar{\delta}$) were respectively 3400, 372, 68 and 47d.p.s./μmol of bile pigment. For pigments 9 and 17 no significant exchange was demonstrated. The somewhat higher radioactivity associated with band 7 probably derives from contaminating labelled material. In another experiment dog bile and [$^{14}C_8$]bilirubin were incubated for 30min at 37°C at pH 9.0. Specific radioactivities were zero for bands 5, 9 and 17, 61d.p.s./μmol for band 7 and 2200 for band 1 ($\bar{\alpha}_0$–$\bar{\alpha}_0$).

Discussion

Structures of isolated bile pigments

Formulation of the bile pigments under investigation as mono- and di-esters of bilirubin-IXα or as the free acid (Scheme 1) accounts satisfactorily for the observations reported above.

*Evidence supporting the bilirubin-*IXα *nucleus.* It is classically admitted that bilirubin from human and animal sources has predominantly the IXα-structure, with the IXβ- and IXδ-structures accounting for at most a few percent of total bile pigment (Petryka, 1966; Nichol & Morell, 1969; O'Carra & Coleran, 1970; Kuenzle, 1970*a*; Tipton & Gray, 1971; Lathe, 1972). Pigment 11 ($\bar{\alpha}_F$–$\bar{\beta}_x$) was identified as bilirubin-IXβ and/or -IXδ (N. Blanckaert, unpublished work); it was well separated from reference bilirubin that moved chromatographically as the most mobile component (Fig. 1).

Recent demonstration of variable and sometimes appreciable amounts of the IIIα and XIIIα structures in commercial bilirubin preparations (McDonagh & Assisi, 1971) and of isomeric transformation of the IXα into equimolecular mixtures of IIIα- and XIIIα-bilirubin in acidic organic solution (Bonnett & McDonagh, 1970) and in aqueous solution (McDonagh & Assisi, 1972) raises two questions: (*a*) do the IIIα- and XIIIα- isomers occur in nature, and (*b*) does isomerization occur in the course of isolation procedures? Hydrolysis of human and rat bile in strongly alkaline medium produced exclusively bilirubin-IXα (Kuenzle, 1970*a*; McDonagh & Assisi, 1972). In such conditions isomerization is inhibited (McDonagh & Assisi, 1972). In the present work the same conclusion was reached for dog bile and was confirmed for human bile. The applied extraction and separation procedures apparently did not induce dipyrrole exchange as (*a*) isolated bile pigments showed the same isomeric composition as untreated dog bile, and (*b*) no significant labelling occurred when [$^{14}C_8$]-bilirubin was preincubated with dog bile followed by extraction and separation of bile pigments, and isolation and analysis of monoconjugates of bilirubin.

A number of observations made with azopigments derived from isolated bile pigments are logically explained on the basis of the postulated IXα structure. The dipyrrolic chromophore shown in Scheme 1 is supported by similarity of the absorption spectra of the azopigments with that of authentic azodipyrrole excluding, in particular, any extensive contamination by mesoazodipyrrole. That vinyl and isovinyl azodipyrrole nuclei (with their proper alignment of methyl, vinyl and propionic acid side chains) are present is indicated by chromatographic separation into two components moving as authentic vinyl and isovinyl isomers of azodipyrrole and of a variety of derivatives of known structure (azodipyrrole methyl ester derived either from azodipyrrole itself or, by methanolysis, from conjugated azopigments; the acetates of azopigments α_2 and α_3). Further, formation from some bile pigments of two azopigments in ratios near to 1 (Table 4) and, more particularly the observed vinyl/isovinyl isomer ratios (Tables 3, 4 and 6) are compatible with the postulated aglycone.

*Nature of the conjugating groups and their linkage to bilirubin-*IXα. Easy methanolysis of azopigments α_x, α_1, α_2, α_3, γ and δ, derived from isolated bile pigments, with the formation of azodipyrrole methyl ester, indicates binding of the conjugating groups in ester linkage to the carboxyl groups of azodipyrrole demonstrating similar linkage in the parent tetrapyrrolic compounds.

The quantitatively most important pigments (Fig. 1) yielded combinations of azopigments

α_0, α_2, α_3 and δ, in agreement with predominance of the same set of derivatives when dog bile is treated directly with diazotized ethyl anthranilate (Fevery *et al.*, 1971). Azopigments α_0, α_2 and α_3, isolated from dog bile, have been identified as azodipyrrole, azodipyrrole (β?)-D-xylopyranoside and azodipyrrole β-D-glucopyranoside respectively (Compernolle *et al.*, 1971). The δ-azopigment isolated from normal rat is the azodipyrrole β-D-glucuronide (Compernolle *et al.*, 1970).

The structures of the preparations of azopigments α_2 and α_3 were established by separation of their acetates into pairs of spots moving as the vinyl and isovinyl isomers of acetylated xylose and glucose conjugates respectively. Formation on methylation, from the δ-preparations, of one predominant derivative moving as the methyl ester of reference azodipyrrole β-D-glucuronide strongly supports the conclusion that the unknown preparations are also glucuronides. Taking account of the divergent views of Kuenzle (1970*b*) and of our own group (Heirwegh *et al.*, 1970; Compernolle *et al.*, 1970) with regard to the nature of the δ-material a final conclusion will be postponed until the question is clarified.

For azopigments α_x and α_1 the presence of conjugating groups bound in ester linkage is indicated by the formation of azodipyrrole methyl esters on alkaline methanolysis. By suitable selection of the chromatographic solvent system α_x and α_1 are easily distinguished from azodipyrrole (Table 5). Acetate formation (Table 7) and lack of reaction with diazomethane are compatible with structures containing hydroxyl and/or amino groups. Azopigment α_x compared with azodipyrrole showed a reversal of the chromatographic mobilities in different solvent systems (Table 5). This behaviour has also been observed for the amide of azodipyrrole but, in contrast, the latter pigment did not react with acetylating agent. The properties of azopigments α_3 and γ, derived from minor and infrequently observed bile pigments, have not been studied in any detail.

Number of conjugating groups and their position(s) relative to the dipyrrole halves of the bilirubin-IXα aglycone. Considering the pigments that were observed in all bile samples, four types of structures can be recognized (Scheme 1; Table 2): (*a*) unconjugated bilirubin (component 1), (*b*) monoconjugated bilirubins (components 5, 7, 9 and 17), (*c*) mixed diconjugates of bilirubin (components 13, 18 and 19), and (*d*) homogeneous diconjugates of bilirubin (components 8, 12, 14 and 20).

Structure assignment in *a* and *d* rests on the formation of equimolecular amounts of vinyl and isovinyl isomers either of azodipyrrole (*a*) or of conjugated azodipyrrole (*d*) (Table 3). The evidence with regard to component 8 ($\bar{\alpha}_1$–$\bar{\alpha}_1$) is somewhat weaker as significant amounts of azopigments α_0 and α_3 were found. However, as these azopigments were present in approximately equimolecular amounts and as the degree of admixture varied strongly from one experiment to the other, contamination of band 8 ($\bar{\alpha}_1$–$\bar{\alpha}_1$) with material from band 9 ($\bar{\alpha}_0$–$\bar{\alpha}_3$) is likely (Fig. 1).

In *b* and *c* identification follows from (1) the formation of equimolecular amounts of two different azopigments, and (2) vinyl–isovinyl isomer analysis (Table 4). The latter analyses also demonstrate about equally important attachment of the conjugating groups to the vinyl and isovinyl dipyrrole halves of the bilirubin-IXα aglycone. Exceptions to this rule were formed by two of seven preparations of band 5 ($\bar{\alpha}_0$–$\bar{\alpha}_x$) that, apparently, were conjugated asymmetrically (cf. Tables 4 and 6).

General comments

The order of migration of the bile pigments (Fig. 1) is in qualitative agreement with prediction from the relative t.l.c. mobilities of the dipyrrolic azo-derivatives (Fig. 2), suggesting that the conjugating groups, studied indirectly in their attachment to dipyrrolic derivatives are present in the isolated bile pigments. Previous kinetic analysis of azopigment formation led to a similar conclusion (Fevery *et al.*, 1971); in the latter studies if loss of a fragment from aglycone or conjugating groups or another change had escaped detection it should have occurred during treatment with diazo-reagent at pH 2.8 for less than 2.5 min. Obviously, if present, the hypothetical structures should indeed be extremely acid-labile.

Bile pigments yielding almost any possible paired combination of the following azopigments were isolated from dog bile: azodipyrrole (α_0), azopigment α_1, azodipyrrole monoxyloside (α_2), azodipyrrole monoglucoside (α_3) and azopigment δ (presumably azodipyrrole monoglucuronide) (Scheme 1, Table 2). One may wonder by what mechanism the conjugates could have been synthesized *in vivo*. If monoconjugates would arise from dipyrrole exchange between bilirubin-IXα and diconjugates of it then one would expect considerable proportions of the IIIα and XIIIα structures in the aglycones. This is excluded by demonstration that the IXα structure predominates in bile. A similar reasoning applies to the possible origin of the mixed diconjugates of bilirubin. Synthesis of bilirubin conjugates by sequential attachment of conjugating groups donated by UDP-sugars is more likely. Formation of homogeneous diconjugates of bilirubin *in vivo* probably occurs via the monoconjugates (Jansen, 1972; Fevery *et al.*, 1972*a*; Heirwegh *et al.*, 1973*b*). Mixed diconjugates of other aglycones do occur in Nature (Layne, 1970). According to Jansen & Stoll (1971) formation *in vitro* of bilirubin monoglucuronide involves glucuronyl transfer to either carboxyl group of the acceptor substance.

Splitting of the components 17 ($\bar{\alpha}_0$–$\bar{\delta}$), 19 ($\bar{\alpha}_3$–$\bar{\delta}$) and 20 ($\bar{\delta}$–$\bar{\delta}$), observed in some analyses with the most hydrophilic solvent system, is of interest. However, for the present, the resolution in this region is not sufficient to demonstrate unequivocally multiplicity of these bands. It is expected that the methods presented here for separation and structure elucidation of bilirubin conjugates may be applicable to other biological fluids, species and pathological states. They should facilitate detection of minor components such as ($\bar{\alpha}_0$–$\bar{\alpha}_x$) and may be useful in attempts to prepare conjugated intermediates for biochemical investigations. As it stands the procedure is not recommended for quantitative evaluation of bile composition as incomplete extraction yields must decrease recovery of the more polar compounds.

Our thanks are due to Dr. J. De Groote and Dr. G. J. Dutton for their encouragement and critical advice, to Dr. N. Blanckaert for permission to discuss unpublished material, and to Dr. J. Van der Stock, Dr. J. De Schepper and Dr. P. Valembois for kindly providing bile samples. The financial support of the Fonds voor Wetenschappelijk Geneeskundig Onderzoek and of the Fonds voor Collectief Wetenschappelijk Onderzoek of Belgium is much appreciated.

References

Billing, B. H., Cole, P. G. & Lathe, G. H. (1957) *Biochem. J.* **65**, 774–784

Black, M., Fevery, J., Parker, D., Jacobson, J., Billing, B. H. & Carson, E. R. (1974) *Clin. Sci. Mol. Med.* **46**, 1–17

Bonnett, R. & McDonagh, A. F. (1970) *Chem. Commun.* 238–239

Cohen, B. J. (1960) *Proc. Soc. Exp. Biol. Med.* **103**, 122–124

Cole, P. G., Lathe, G. H. & Billing, B. H. (1954) *Biochem. J.* **57**, 514–518

Compernolle, F., Jansen, F. H. & Heirwegh, K. P. M. (1970) *Biochem. J.* **120**, 891–894

Compernolle, F., Van Hees, G. P., Fevery, J. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 811–819

Fevery, J., Van Hees, G. P., Leroy, P., Compernolle, F. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 803–810

Fevery, J., Leroy, P., Van de Vijver, M. & Heirwegh, K. P. M. (1972*a*) *Biochem. J.* **129**, 635–644

Fevery, J., Van Damme, B., Michiels, R., De Groote, J. & Heirwegh, K. P. M. (1972*b*) *J. Clin. Invest.* **51**, 2482–2492

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509

Gordon, E. R., Dadoun, M., Goresky, C. A., Chan, T.-H. & Perlin, A. S. (1974) *Biochem. J.* **143**, 97–105

Görges, Th. & Gohr, H. (1954) *Deut. Z. Verdau. Stoffwechseikr.* **14**, 187–188

Heirwegh, K. P. M., Van Hees, G. P., Leroy, P., Van Roy, F. P. & Jansen, F. H. (1970) *Biochem. J.* **120**, 877–890

Heirwegh, K. P. M., Fevery, J., Meuwissen, J. A. T. P., De Groote, J., Compernolle, F., Desmet, V. & Van Roy, F. P. (1973*a*) *Methods Biochem. Anal.* **22**, 205–250

Heirwegh, K. P. M., Meuwissen, J. A. T. P. & Fevery, J. (1973*b*) *Advan. Clin. Chem.* **16**, 239–289

Jansen, F. H. & Billing, B. H. (1971) *Biochem. J.* **125**, 917–919

Jansen, F. H. & Stoll, M. S. (1971) *Biochem. J.* **125**, 585–597

Jansen, P. L. M. (1972) *Folia Med. Neerl.* **15**, 207–213

Jansen, P. L. M. (1973) *Clin. Chim. Acta* **49**, 233–240

Kuenzle, C. C. (1970*a*) *Biochem. J.* **119**, 395–409

Kuenzle, C. C. (1970*b*) *Biochem. J.* **119**, 411–435

Lathe, G H. (1972) *Essays Biochem.* **8**, 107–148

Layne, D. S. (1970) in *Metabolic Conjugation and Metabolic Hydrolysis* (Fishman, W. H., ed.), vol. 1, pp. 21–52, Academic Press, New York

McDonagh, A. F. & Assisi, F. (1971) *FEBS Lett.* **18**, 315–317

McDonagh, A. F. & Assisi, F. (1972) *Biochem. J.* **129**, 797–800

Nichol, A. W. & Morell, D. B. (1969) *Biochim. Biophys. Acta* **184**, 173–183

O'Carra, P. & Coleran, E. (1970) *J. Chromatogr.* **59**, 458–468

Ostrow, J. D. & Murphy, N. B. (1970) *Biochem. J.* **120**, 311–327

Ostrow, J. D., Hammaker, L. & Schmid, R. (1961) *J. Clin. Invest.* **40**, 1442–1452

Petryka, Z. J. (1966) *Proc. Soc. Exp. Biol. Med.* **123**, 464–466

Schmid, R. (1957) *J. Biol. Chem.* **229**, 881–888

Stoll, M. S. & Gray, C. H. (1970) *Biochem. J.* **117**, 271–290

Talafant, E. (1956) *Nature (London)* **178**, 312

Tipton, G. & Gray, C. H. (1971) *J. Chromatogr.* **59**, 29–43

Wren, J. J. & Szszepanowska, A. D. (1964) *J. Chromatogr.* **14**, 405–410